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RESEARCH IN PLAGUE IMMUNITY (U)

FINAL REPORT

by

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Final Report to The U.S. Army Medical Research and Development Command

SUMMARY

The observations reported here traced the development of the Fraction I envelope antigen ultrastructurally in both virulent and avirulent strains of Yersinia pestis grown for varying periods of incubation at 37 C, to study the effects of Ca^{2+} and Mg^{2+} on the production of the envelope antigen and to determine, by passage of Y. pestis through animals, the yield of the pathogen's Fraction I antigen. These findings confirmed the hypothesis that the yield of extracellular substance (Fraction I) was higher under the abovementioned conditions. As a result, a better antigenic culture of Yersiniae for the immunoprophylaxis of plague was prepared.

Subsequently, the virulent Y. pestis, grown on heart infusion blood agar, was examined by scanning electron microscopy: the Fraction I envelope antigen can be visualized as a lumpy coating on the surface of the bacilli. The amount of antigen is dependent on both length and temperature of incubation. A longer period of incubation (72 h) at a higher temperature (37 C) yielded more Fraction I antigen. The virulent organism Yersinia pseudotuberculosis, closely related to Y. pestis, exhibited no such coating at any temperature and incubation period. The presence of flagella was demonstrated when this organism was grown at 22 C, which is diagnostically valuable in distinguishing it from Y. pestis.

A live, attenuated Y. pestis vaccine strain EV76 (Paris) F was studied for safety and immunogenicity. This vaccine was orally administered to the highly susceptible animal species C. aethiops (the vaccine strain is pathogenic in C. aethiops when administered parenterally). The efficacy of the vaccine given orally in prevention of bubonic plague was ascertained in this experiment.

Protection against pneumonic plague by the oral administration of the same live, avirulent Y. pestis was also evaluated in the highly susceptible Cercopithecus monkeys. We found the live, attenuated EV76 (Paris) F, orally-administered to be very effective in preventing pneumonic plague. Therefore, so far as we are aware, it is probably the simplest and most efficacious weapon against pneumonic plague, as we have shown in our limited but significant trial study.

From time to time, certain bacteriological controversies have interested medical historians. Often such disputes concern the priority of discovery, but sometimes the content of the discovery is at question. The case in the point is the independent observation of the plague bacillus by Alexandre Yersin and Schibaburo Kitasato in 1894. There seems to be not the slightest doubt that the organisms described by both Yersin and Kitasato are the same. However, most textbook writers, still confused by the vast literatures, are not in agreement when ascribing credit for this discovery. Bibel and Chen recognised the complexity of this problem and have therefore subjected the material to a critical review to clarify the long-standing confusion as to who really discovered the plague bacillus.

A study of the cellular immune response to the plague bacillus, Yersinia pestis, indicates that resistance to infection is dependent on the ability of the macrophage to inactivate phagocytized bacteria and withstand their cytotoxic effect. Serum from Y. pestis immunized mice 1) enhanced the resistance of normal PE* monolayers to the cytotoxicity; 2) increased inactivation of bacteria by primed PE cells. The enhancing serum component was not removed by absorption with heat-killed Y. pestis. A similar enhancement provided by supernatant fluids of spleen cultures from immunized but not nonimmunized mice was reduced by pretreatment of spleen cells with rabbit anti-(mouse brain) hyperimmune serum plus complement and by removal of the glass adherent cell population. Thus, the enhancing serum component appears to be a product(s) of thymus-derived lymphocytes which, when primed, require splenic microphages for the interaction with heat-killed Y. pestis.

* Peritoneal exudate

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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I. Yersinia pestis: Correlation of Ultrastructure and Immunological Status.

The objectives of the research are three: to trace the development of the fraction I envelope antigen ultrastructurally in virulent and avirulent strains of Y. pestis, to study the effects of certain chemical agents and animal passage on antigen production, and to evaluate the antigen semi-quantitatively, correlating our results with our ultrastructural findings.

Materials and Methods

The following strains of Y. pestis were used in this study: Wild-type Tl, EV76 (Paris) F, EV76 (Paris) R, EV 406, and M 23. They were studied ultrastructurally and for localization of fraction I "envelope" antigen. Lyophilized stocks were transferred to blood or heart infusion agar for ultrastructural or biochemical studies. Cultures were sampled after 24, 48 and 72 h at 37 C. Cell-to-cell relationships were preserved by glutaraldehyed-fixation of colonies in situ. Best fixation of the fraction I cell envelope antigen and greatest general ultrastructural preservation were achieved by staining with ruthenium red, post-fixing in osmium tetroxide, staining en bloc with uranyl acetate, embedding in Spurr's resin mixture, sectioning and staining with ethanolic uranyl acetate and lead citrate. Semi-quantitative reverse CF determination of envelope fraction I were carried out on dilutions of the supernate of centrifuged acetone-killed and dried, saline-extracted 72 h organisms.

Results

Ultrastructural studies: Virulent strains yielded more envelope antigen than avirulent strains with the exception of the attenuated EV series whose immunogenicity and virulence had been purposely enhanced by injection through a series of iron sulfate-injected guinea pigs. Ultrastructure in all strains was similar and resembled other well-preserved gram-negative bacteria. The bacterial wall consisted of a densely staining, tightly adherent cell surface coat surrounding the cell wall membrane which in turn surrounds the R or peptidoglycan layer: the cell wall follows the contours of the membrane with a uniform space imposed between the two layers. Internal cellular organelles are typical of gram negative organisms but with a diffuse nucleoid area which was undivided from the ribosome-rich region. Age and strain differences were reflected in the envelope material which was fibrous or floccular. Ultrastructural differences from previously reported studies are a result of fixation: ruthenium red fixation appears to prevent commonly observed deleterious effects of en bloc staining and to allow better preservation of ultrastructure.

Cells of 24 h cultures of wild type Y. pestis were surrounded by an extensive extracellular fibrillar matrix resembling the envelope antigen; with avirulent EV strains, the extracellular matrix appeared after 24 or 72 h of incubation. The strain was grown in the presence

of Ca^{2+} and Mg^{2+} was also studied. Poorly immunogenic strain EV 406 had little if any extracellular matrix after 72 h of growth until it was passed through a guinea pig. Consistant with a lack of an envelope, the fraction I negative mutant strain M 23 lacked the extracellular matrix at 48 h and showed little at 72 h.

Assay of envelope antigen content: Fixation reactions of extracts of 72 h cultures of Y. pestis were positive in EV76 (Paris) R and F and indicated that passage through guinea pigs was essential to production of envelope antigen, especially noted in strain EV 406.

Discussion

The major immunogen in human Y. pestis infections are components of the bacterial envelope. The envelope can be seen in electron micrographs of virulent strains after 24 h of growth but are not seen in immunogenic avirulent strains until after 48 h of growth or after animal passage. This suggests that virulence is related to rapid production of the envelope material which is probably associated with resistance to phagocytosis during the first 24 h of infection.

Animal passage enhances virulence and selectively heightens immunogenicity. Addition of Ca^{2+} and Mg^{2+} to media of virulent Y. pestis cultures also increases antigenicity and appears to be necessary for production of the extracellular matrix. Semi-quantitative determination of fraction I content in supernatant fluids confirm ultrastructural evidence that this matrix is the source of the envelope antigen.

II. Scanning Electron Microscopic Study of Virulent Yersinia pestis and Yersinia pseudotuberculosis Type I.

The objectives of the study were to visualize the fraction I envelope antigen on the Y. pestis cell surface by scanning electron microscopy and to use scanning electron microscopy to discriminate between Y. pestis and the closely related bacterium Y. pseudotuberculosis.

Materials and Methods

Lyophilized Yersinia stocks maintained at 4 C were transferred to heart infusion blood agar slants and incubated at 22 C and 37 C for 24, 48 and 72 h for Y. pestis, and at 22 C in heart infusion broth for 48 h for Y. pseudotuberculosis. Cultures were glutaraldehyde-fixed in situ, transferred to polylysine-coated coverslips, rinsed, dehydrated in ethanol and Freon 113, critical-point dried in Freon 13 and coated with gold-palladium.

Results and Discussion

Virulent Y. pestis T1, grown at 22 C at different incubation times, yielded small amounts of fraction I envelope antigen on the surface identified as a light, particulate coating on the bacterial surface and by particles on the surrounding surfaces at 72 h growth. The same strain grown at 37 C produces an abundant rough granular particulate substance within 24 h which increased with incubation time, particles and particle aggregates spilling onto the surrounding surfaces. This antigen envelope is water soluble necessitating careful in situ fixation of cultures before washing them.

Differentiation of Y. pestis from Y. pseudotuberculosis is important in quiescent or uninfected areas and is difficult because of their similar morphology by light microscopy and their serologic similarities. Demonstration of the surface flagella of Y. pseudotuberculosis is both faster and more reliable than transmission electron microscopy, suggesting a useful diagnostic tool.

III. Immunity in Plague: Protection Induced in Cercopithecus aethiops by Oral Administration of Live, Attenuated Yersinia pestis.

The objectives of this study are to examine the efficacy, safety and immunogenicity of orally administered Y. pestis EV76 (Paris) F in Cercopithecus aethiops (a highly susceptible host) in the prevention of both bubonic and pneumonic plague.

Materials and Methods

Organisms. The virulent Indian isolate 195/P of Y. pestis for challenge infection was grown in heart infusion broth supplemented with $CaCl_2$, $MgCl_2$ and xylose at 37 C for 24 h in a rotary shaker before subculture. Viability was tested on blood agar base plates and purity, by specific bacteriophage testing.

The vaccine strain, EV76 (Paris) F or Y. pestis was passaged in guinea pigs pretreated with non-toxic doses of iron salts.

Preparation of vaccine. EV76 (Paris) F was grown on heart infusion agar supplemented with $CaCl_2$, $MgCl_2$ and xylose in Roux bottles at 37 C for 72 h and harvested in heart infusion broth. A commercially available killed plague vaccine (Cutter Laboratories, Berkeley, California) was also used.

Autopsies and serology. Spleen impression smears were carefully studied and spleen and cardiac blood were cultured for Y. pestis at autopsy. Vaccine induced antibody was demonstrated by CF and passive HA tests using plague-specific fraction I as the antigen.

Results

Three vervets were given 10^6 viable organisms of the vaccine strain in a piece of banana; and three, 10^9. Orally administered, EV76 (Paris) F was less toxic than when administered subcutaneously but three monkeys had a high transient fever and gastrointestinal disturbances during the third week after infection, recovering within four days. All monkeys except one inoculated with 10^9 organisms remained seronegative for HA and CF antibody to fraction 1.

Intradermal challenge with a high dose of pathogenic organisms resulted in death from bubonic plague of all seronegative monkeys within 7 days. Survival of the seropositive monkey indicated that the vaccine was protective but a single dose of 10^9 organisms was probably not adequate.

Repeated oral administration of the vaccine strain in banana to a new series of 6 monkeys results low levels of plague-specific antibody in only 2 animals until the method of delivery was changed to allow greater contact of the organisms with the oral mucosa. Organisms administered in a disposable TB syringe sans needle to partially anaesthetized monkeys caused seroconversion 2 weeks after vaccination. One seropisitive monkey revaccinated twice with the Cutter formalin-killed vaccine still had only low levels of circulating antibody.

Intradermal challenge with a high dose of the virulent strain, 195/P, showed that the seropositive animals were protected whereas unvaccinated controls died within a week. Autopsy studies of fatal infections revealed classical signs of bubonic plague: wide spread cutaneous hemorrhage, abdominal edema and edematous tissues surrounding the bubonic lesions in the enlarged axillary lymph nodes.

Discussion

Experiments indicate that orally administered EV76 (Paris) F is non-lethal to vervets and that involvement of the oral mucosa and production of circulating CF and HA antibody are necessary for induction of protective immunity. Severity of the gastrointestinal reaction to the vaccine strain was not an indication of protection. Despite gradually declining antibody titers during the 11-12 weeks post-inoculation animals resited a virulent challenge strain, suggesting a booster effect of the challenge dose on a potential immune response.

This research implies that the vaccine is probably safe to administer to humans since, although some of the test animals become ill, none died; furthermore, humans are slightly less susceptible than non-human primates. The method of delivery to humans is probably of less importance since retention of the vaccine in the mouth for a sufficient period of time is easily achieved.

IV. Immunity in Plague: Protection of Vervet (Cercopithecus aethiops) Against Pneumonic Plague by Oral Administration of Live, Attenuated Yersinia pestis.

The objectives of this study were to further verify the safety and efficacy of peroral vaccination in inducing immunity against aerosol induced pneumonic plague.

Material and Methods

Organisms and animals. The vaccine strain EV76 (Paris) F of Y. pestis was administered orally while the virulent strain Y. pestis 195/P was employed in aerosol infections; both strains were handled as previously described. Nine male vervets (C. aethiops) weighing 3.95-4 kg (at the time challenge) constituted the study animals.

Immunization and serological analysis. Peroral vaccination of the semi-anaesthetized vervets was accomplished using a tuberculin syringe (without needle) to drop 1.175×10^9 cfu (in 0.5 ml) of the vaccine strain into the mouth; deglutitive reflexes occurred shortly thereafter. Rectal temperatures as well as the general state of the animals' health were recorded daily for 2 weeks. Blood samples were taken weekly for serological tests for vaccine-induced CF or HA antibody titers to plague-specific fraction I antigen.

At 15 weeks post-vaccination, anaesthetized monkeys were exposed to an aerosol challenge of virulent Y. pestis strain 195/P in a modified Henderson apparatus. Aliquots of the aerosol taken at 5 minute intervals during the 20 minute exposure period and cultured on blood agar base medium were used to determine the cfu of Y. pestis in the inhaled volume as 0.32 ml/g body weight/min. After exposure, monkeys were air-washed for 15 min. and removed to a holding room at 70-75 F where stringent safety precautions were observed in their handling.

Necropsies of fatal infections were limited to gross examinations and cultures of lung and heart blood and impression smears of various organs. Recovery of Y. pestis was confirmed by bacteriophage typing.

Results

Antibody response to the vaccine strain. Six vervets tolerated a single dose of EV76 (Paris) F with no local or systemic reaction except for a slight loss of appetite 7-10 days post-inoculation in 3 animals. Within 7 days all 6 animals had primary HA titers ranging from 1:8 to 1:64 reading maximum levels (1:128-1:1,024) between the second and third weeks post-immunization, declining gradually until the twelfth week and dropping to 1:16-1:128 by the fourteenth. CF antibody levels showed the same pattern but appeared a week later. The two monkeys having the lowest antibody titer later succumbed to the challenge infection.

Protective efficacy of peroral vaccine. The challenge dose (3.2×10^6 - 4.9×10^6 organisms) of Y. pestis strain 195/P inhaled at 15 weeks post-vaccination by the monkeys was extremely high considering that the LD_{50} by the intradermal route is estimated to be as little as 5-50 cfu for vervets; and for Macaca mulatta, 1×10^2 intratracheally and 2×10^4 by aerosol. The two monkeys with the lowest antibody levels and one with a fair antibody titer as well as 3 nonimmunized controls succumbed to severe pneumonic plague on the fourth day post-exposure to the aerosol challenge.

Three survivors showed no signs of pathology when sacrificed at day 36 post-exposure; antibody levels were high (1:64-1:128 by passive hemagglutination and 1:32-1:128 for complement fixation).

Fatal infections demonstrated widespread lung consolidation, thoracic and perocardial effusions, pale, friable myocardium with petechial hemorrhages, and distention of the right side of the heart with poorly clotted blood in the cavity. Cervical axillary nodes in two monkeys. The abdominal cavities appeared to be consistently unchanged, but the spleens were somewhat enlarged, with markedly rounded edges. Although the livers were moderately enlarged, their margins were sharp; however, early fatty degeneration was evident. Adrenal cortices exhibited meager hemorrhage; kidneys were pallid. Lung and heart blood yielded confluent growths of Y. pestis except for negative blood cultures in 2 immunized animals. Lung smears yielded abundant plague bacilli in all 6 monkeys.

Discussion

On the basis of these findings, avirulent, live orally-administered EV76 (Paris) F can be considered effective in the prevention of bubonic and pneumonic plague though prophylactic aspects of administration of Y. pestis and the dosages await further confirmation. Lack of severe symptoms to the vaccine strain both in this study and the previous one indicate that the vaccine is safe in vervets. Elimination of behavioral variables related to vaccine administration allowed consistent production of vaccine-induced HA and CF antibodies within 1-2 weeks which invariably accompanied protection. It is significant that antibody titers in fact correlated with protective immunity and therefore represent a reliable indicator of anti-plague immunity even against the pneumonic form. The inhaled challenge dose reported here was much larger than intended, suggesting that more animals would have survived a smaller challenge; previous reports from the literature support this supposition. Moreover, there is only one report in the literature of successful vaccination against pneumonic plague. This was accomplished by "hyperimmunization" of rhesus monkeys by a complex vaccination schedule involving administration of $4\text{-}5 \times 10^9$ cfu of EV76 in 4-5 properly spaced doses by 5 different routes over a period of 3 months. Such "hyperimmunization" schedules are hardly practical for mass field immunizations. Live, attenuated EV76 (Paris) F administered orally is probably the simplest, most efficious weapon against pneumonic plague.

V. The Diagnosis of Plague: An Analysis of the Yersin-Kitasato Controversy.

A bacteriological controversy over priority and content of discovery which has interested historians for almost a century is that concerning the independent discovery of the plague bacillus by Alexandre Yersin and by Shibasaburo Kitasato in Hong-Kong in 1894. There is no doubt that Yersin

correctly described and cultured Yersinia pestis, the etiological agent of plague; the question is, whether Kitasato did.

Yersin correctly described the bacillus in June of that year as a thick, gram-negative bipolar-staining (with aniline dyes) bacillus with rounded ends which could be isolated from buboes of plague victims and in severe cases, form blood. He discovered involution forms and studied colony morphology on agar (transparent white with irridescent edges under reflected light). He inoculated animals and studied pathogenesis in susceptible hosts (mice, rats and guinea pigs but not pigeons).

Kitasato's first report in August was hastily written and ambiguous, stating that the bacillus was similar to an encapsulated diplococcus. A later, preliminary bacteriological study describes the bacillus as being similar to Yersin's bacillus with a few exceptions. Kitasato did not commit himself on the gram reaction, he mentioned slight motility and slight turbidity in broth. In addition, his temperature studies were contradictory and his description of colony formation was somewhat different (whitish-gray with a blueish appearance by reflected light). He also performed animal inoculations and tested Koch's postulates, noting lymph node involvement and appearance of disease organisms in blood.

Conflicting statements by Kitasato's coworkers, and concessionary statements by Kitasato himself shed some doubt on the validity of Kitasato's claim. However, since preparations isolated by both researchers were comparable on examination (except for one of Kitasato's blood smears) there is little doubt that Kitasato did originally isolate the etiological agent of plague. The authors note that there are three possibilities: 1) that Kitasato did not observe and isolate Y. pestis but a pneumoncoccus-like bacterium; 2) that he observed plague but initial cultures were of a pneumococcus or mixed; or 3) that he observed and isolated plague but subsequently contiminated his subcultures, or isolated a different organism later. The third possibility is favored by the authors and it is supported by variations in colony morphology, in subjectivity of judgements by investigators, lack of genetic homogeneity of laboratory animals, difficulties with unreliable reagents, lack of appreciation of the importance of standardized methodology, inoculum concentration dosage effects on temperature and animal pathogenesis studies. pleomorphism of the plague bacillus and its sensitivity to the environment, common secondary infections of plague victims with pneumococcus, not conductive to careful, well-planned and executed work. The authors believe that Kitasato should have an equal share in the claim of first discovery of Yersinia pestis with Yersin.

VI. Cellular Immune Response to Yersinia pestis Modulated by Product(s) from T cells.

A study of the cellular immune response to the plague bacillus, Yersinia pestis, indicates that resistance to infection is dependent upon whether the macrophage can inactivate and withstand the cytotoxic effects of phagocytized bacilli. Serum from mice immunized with antigens of Y. pestis 1) enhanced the resistance of monolayers of normal cultures to the cytotoxic

effects of Y. pestis and 2) increased the capacity of PE cells from immune mice to inactivate these bacteria. The enhancing serum component was not removed by absorption with heat-killed Y. pestis. Similar enhancement was provided by supernatant fluids of spleen cultures (Mishell and Dutton) from immunized but not nonimmunized mice. Pretreatment of spleen cells with rabbit anti-(mouse brain), hyperimmune serum plus complement caused a reduction in the enhancing capacity of their culture fluids. Removal of the glass adherent cell population from primed spleen cell suspensions prior to antigenic stimulation in vitro resulted in a loss in activity from subsequently harvested culture fluids. Thus, the enhancing serum component appears to be a product(s) of thymus-derived lymphocytes. Furthermore, splenic microphages seem required for the interaction of primed T cells with heat-killed Y. pestis.

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T. H. Chen, Sanford S. Elberg, and Daniel M. Eisler. Immunity in Plague: Protection of the Vervet (Cercopithecus aethiops) Against Pneumonic Plague by the Oral Administration of Live, Attenuated Yersinia pestis Submitted to the J. Infect. Dis. for publication. (April 1976)

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John F. Wong, and Sanford S. Elberg. Cellular Immune Response to Yersinia pestis Modulated by Product(s) from T. Cells. Submitted to the J. Infect. Dis. for publication. (March 1976)

LIST OF PERSONNEL RECEIVING CONTRACT SUPPORT

T. H. Chen, M.D.		
	Research Microbiologist	1 November 1973 to 15 March 1976
John F. Wong, Ph.D. (Ph.D. received in 1975)		1 November 1973 to 30 June 1975

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The ultrastructure of the plague bacillus and its relationship to immunity were studied by electron transmission and scanning electron microscopic methods. In electron micrographs of colonies, the extracellular material is clearly present after 24 h of growth in virulent strain cultures but not in avirulent strain EV76 (Paris) R. The material did appear in EV76 (Paris) R, however, after 48 h of culture, as well as in once-passaged EV 406. With the addition of Ca^{2+} and Mg^{2+} to the medium virulent Y. pestis, grown at 37 C, acquired more

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Antigenic components.

When virulent Y. pestis, grown on heart infusion blood agar, is examined by scanning electron microscopy, the fraction I envelope antigen can be seen as a lumpy coating on the surface of the cells which spills over to the surrounding surface. The amount of this antigen is dependent on both length and temperature of incubation.

Experiments on highly susceptible nonhuman primates (C. aethiops) with avirulent strain EV76 (Paris) F given orally demonstrated 1) that the vaccine strain is nonlethal; 2) that the vaccine prevents both bubonic and pneumonic infections by challenge with a fully virulent strain of Y. pestis.